invitrogen™

BioModule™ Western Analysis

For protein analysis and detection

Catalog nos. WFGE09, WFGE10

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User Manual

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Experienced Users Procedure

Introduction

A brief experienced user's procedure for performing western blotting and immunodetection is described below. For a detailed protocol, refer to the manual.

Step	Procedure
Prepare Lysates	Prepare mammalian cell (10^7-10^8 cells) or tissue (100 mg) lysate on ice using 1 ml **fresh** Cell Extraction Buffer with 1:20 Protease Inhibitor Cocktail (see page 9 for details). If you have prepared lysates using another method, proceed to the next step.
Prepare Sample and Buffer	Prepare protein sample at the appropriate concentration using the NuPAGE® LDS Sample Buffer and NuPAGE® Sample Reducing Agent (for reduced samples) at a final concentration of 1X. Prepare 1 L of 1X NuPAGE® MES SDS Running Buffer. See page 12 for details.
Prepare Gel Cassette	1. Remove the gel cassette from the pouch and rinse with deionized water. 2. Peel off the tape covering the slot on the back of the gel cassette and pull the comb out of the cassette. Rinse the wells with 1X Running Buffer and fill sample wells with 1X Running Buffer.
Perform Electrophoresis	1. Assemble the Buffer Core with two gel cassettes or one gel cassette and a Buffer Dam. Insert the Buffer Core with gels into the Mini-Cell and lock the Gel Tension Wedge. 2. Fill the Upper Buffer Chamber with 200 ml 1X Running Buffer (be sure to check for leaks before adding the entire amount). For **reduced** samples, fill the Upper Buffer Chamber with 200 ml of 1X Running Buffer containing 0.5 ml NuPAGE® Antioxidant. 3. Load 10-25 µl sample at the desired protein concentration onto each well of the gel. 4. Pre-mix 10 µl MagicMark™ XP Western Standard with 5 µl SeeBlue® Plus2 Pre-stained Standard. Load 15 µl of the protein standard mixture onto a well. 5. Fill the Lower Buffer Chamber (anode) with 600 ml 1X Running Buffer. 6. Place the lid on the assembled XCell *SureLock*™ Mini-Cell and align the electrodes to firmly seat the lid onto the Mini-Cell. 7. With the power **off**, connect the electrode cords to power supply, turn on the power, and perform electrophoresis at **200 V Constant for 35 minutes** (start current: 110-125 mA and end current: 70-80 mA).
Disassembly	1. Remove the lid, discard the buffer, and unlock the Gel Tension Wedge. 2. Remove the Buffer Core with the gel cassettes from the Lower Buffer Chamber while holding the cassettes against the core. 3. Remove gel cassettes from the Buffer Cores and open the gel cassette using the Gel Knife by pushing up and down gently on the knife's handle to separate the plates. 4. Proceed to the Western transfer protocol, below.
Preparing for Transfer	1. Prepare 1 L of 1X NuPAGE® Transfer Buffer by adding 50 ml 20X NuPAGE® Transfer Buffer and 100 ml methanol to 850 ml deionized water. Add 1 ml NuPAGE® Antioxidant in the transfer buffer for **reduced** samples. 2. Soak blotting pads in 700 ml 1X NuPAGE® Transfer Buffer and soak the filter paper briefly in 1X NuPAGE® Transfer Buffer. 3. Pre-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol. Briefly rinse in deionized water and then place the membrane in the Incubation Tray containing 50 ml 1X NuPAGE® Transfer Buffer for several minutes.

Continued on next page

Experienced Users Procedure, Continued

<table>
<tr><th>Step</th><th>Procedure</th></tr>
<tr><td>Western Blotting</td><td>Instructions for transferring one gel are described below. To transfer two gels, see page 23.
1. Place a piece of pre-soaked filter paper on top of the gel (adhered to the bottom plate) and remove any trapped air bubbles.
2. Turn the plate over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface. Place a pre-soaked transfer membrane on the gel.
3. Place another pre-soaked filter paper on top of the membrane. Place two soaked blotting pads into the cathode (-) core of the blot module.
4. Carefully pick up the gel/membrane assembly and place on blotting pad such that the gel is closest to the cathode core. Add enough pre-soaked blotting pads to rise to 0.5 cm over rim of cathode core. Place the anode (+) core on top of the pads.
5. Hold the blot module together firmly and slide it into the Lower Buffer Chamber.
6. Insert and lock the Gel Tension Wedge into the Lower Buffer Chamber.
7. Fill the blot module with 1X NuPAGE® Transfer Buffer until the gel/membrane assembly is covered. Fill the Outer Buffer Chamber with 650 ml deionized water.
8. Place the lid on the unit and connect the electrical leads to the power supply.
9. Perform transfer using 30 V constant for 1 hour (Current: start: 170 mA; end: 110 mA).</td></tr>
<tr><td>Western Immuno-detection</td><td>1. Prepare solutions for Western immunodetection as described on pages 29 and 33.
2. Wash the membrane twice with deionized water for 5 minutes each.
3. Block the membrane in 10 ml Blocking Solution. Incubate for 30 minutes on a rotary shaker set at 1 revolution/sec. Decant the Blocking Solution.
4. Rinse with 20 ml water for 5 minutes, then decant. Repeat once.
5. Incubate with 10 ml Primary Antibody Solution for 1 hour, then decant.
6. Wash membrane for 5 minutes with 20 ml Antibody Wash, then decant. Repeat 3 times.
7. Incubate with 10 ml Secondary Antibody Solution for 30 minutes, then decant.
8. Wash for 5 minutes with 20 ml Antibody Wash, then decant. Repeat 3 times.
9. Rinse with 20 ml deionized water for 2 minutes, then decant. Repeat twice.
10. Perform chemiluminescent or chromogenic detection as follows:
<table>
<tr><th>Chemiluminescent</th><th>Chromogenic</th></tr>
<tr><td>• Place the membrane on a sheet of transparency plastic and apply 2.5 ml Chemiluminescent Substrate without touching the membrane surface using a clean pipette. Incubate for 5 minutes.
• Blot any excess solution using the filter paper from the kit.
• Cover the membrane with a piece of transparency plastic and expose X-ray film to the membrane sandwich for 1 second to several minutes.</td><td>• Incubate the membrane with 5 ml Chromogenic Substrate until purple bands develop on the membrane. Development is complete in 1-60 minutes.
• Rinse the membrane three times with 20 ml deionized water for 2 minutes.
• Air-dry the membrane on a clean piece of filter paper, or dry the membrane using a stream of slightly warm air or under an infrared lamp.</td></tr>
</table></td></tr>
</table>

Kit Contents

Types of Products

This manual is supplied with the following products:

Components	Catalog no.
BioModule™ Western Analysis	
with Chromogenic Detection	WFGE09
with Chemiluminescent Detection	WFGE10

Kit Components

The components included with the BioModule™ Western Units are described below. Sufficient reagents are provided to perform 20 western blotting and immunodetection experiments.

Components	Quantity	WFGE09	WFGE10
Cell Extraction Buffer	100 ml	√	√
Protease Inhibitor Cocktail	1 ml	√	√
NuPAGE® LDS Sample Buffer (4X)	10 ml	√	√
NuPAGE® Sample Reducing Agent (10X)	2 x 250 µl	√	√
NuPAGE® Novex 4-12% Bis-Tris Mini Gel (1.0 mm, 10-well)	20 gels	√	√
NuPAGE® MES SDS Running Buffer (20X)	2 x 500 ml	√	√
MagicMark™ XP Western Protein Standard	250 µl	√	√
SeeBlue®Plus2 Pre-Stained Protein Standard	500 µl	√	√
NuPAGE® Transfer Buffer (20X)	1 L	√	√
NuPAGE® Antioxidant	2 x 15 ml	√	√
Invitrolon™ PVDF/Filter Membrane Sandwiches	20 membranes	√	√
WesternBreeze® Chromogenic Kit	1 kit	√	
WesternBreeze® Chemiluminescent Kit	1 kit		√

Continued on next page

Shipping and Storage

The shipping conditions for each component are listed in the table below. Upon receipt, store the components as described below.

Components	Shipping	Storage
Cell Extraction Buffer	Dry ice	-20ºC
Protease Inhibitor Cocktail for Mammalian Tissues	Dry ice	-20ºC
NuPAGE® LDS Sample Buffer (4X)	Room temperature	4ºC to 25ºC
NuPAGE® Sample Reducing Agent (10X)	Blue ice	4ºC
NuPAGE® Novex 4-12% Bis-Tris Mini Gel (1.0 mm, 10-well)	Room temperature	Room temperature
NuPAGE® MES SDS Running Buffer (20X)	Room temperature	4ºC to 25ºC
MagicMark™ XP Western Protein Standard (supplied in loading buffer containing 125 mM Tris-HCl, pH 6.8; 10 mM DTT; 17.4% glycerol; 3% SDS; and 0.025% bromophenol blue)	Dry ice	-20ºC To avoid repeated freeze/thaw, aliquot in small volumes and store
SeeBlue Plus2 Pre-Stained Protein Standard (supplied in loading buffer containing Tris-HCl, Formamide, SDS, Phenol Red)	Blue ice	4ºC
NuPAGE® Transfer Buffer (20X)	Room temperature	4ºC to 25ºC
NuPAGE® Antioxidant	Blue ice	4ºC
Invitrolon™ PVDF/Filter Membrane Sandwiches	Room temperature	Room temperature
WesternBreeze® Chemiluminescent Kit	Blue ice	2ºC to 8ºC
WesternBreeze® Chromogenic Kit	Blue ice	2ºC to 8ºC

Cell Extraction Buffer

The composition of the Cell Extraction Buffer is listed below. Thaw the buffer on ice and after first use, aliquot the buffer into smaller volumes. Store the aliquots at -20ºC to avoid repeated freezing and thawing.

10 mM Tris-HCl, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$ (sodium pyrophosphate)
2 mM Na_3VO_4 (sodium vanadate)
1% Triton X-100
10% Glycerol
0.1% SDS
0.5% deoxycholate

Continued on next page

Protease Inhibitor Cocktail for Mammalian Tissues

The Protease Inhibitor Cocktail is a mixture of the following protease inhibitors and is supplied as a solution in DMSO (dimethyl sulfoxide). Thaw the cocktail quickly at room temperature and after first use, aliquot the cocktail into smaller volumes, and store the aliquots at -20ºC to avoid repeated freezing and thawing.

Inhibitor	Inhibits	Concentration
AEBSF	Serine proteases such as trypsin and chymotrypsin	104 mM
Aprotonin	Serine proteases such as trypsin, plasmin, chymotrypsin, trypsinogen, urokinase, kallikrein, human leukocyte elastase	80 µM
Leupeptin	Serine and cysteine proteases such as calpain, trypsin, papain, cathepsin B	2 mM
Bestatin	Aminopeptidases such as leucine aminopeptidase and alanyl aminopeptidase	4 mM
Pepstatin A	Aspartic proteases such as pepsin (human or porcine), rennin, cathepsin D, chymosin (bovine rennin), and protease B	1.5 mM
E-64	Cysteine proteases such as calpain, papain, and cathepsins B and L	1.4 mM

WesternBreeze® Chromogenic Kit Contents

The components included in the WesternBreeze® Chromogenic Immunodetection Kit are listed below. Sufficient reagents are supplied to detect 20 mini-blots.

Item	Description	Amount
Blocker/Diluent (Part A)	Concentrated buffered saline solution containing detergent	80 ml
Blocker/Diluent (Part B)	Concentrated Hammersten casein solution	80 ml
Antibody Wash (16X)	Concentrated buffered saline solution containing detergent	2 x 100 ml
Chromogenic Substrate	Ready-to-use solution of BCIP/NBT substrate for alkaline phosphatase.	100 ml
Secondary Antibody Solution	Ready-to-use solution of alkaline phosphatase-conjugated, affinity purified, anti-species IgG (one each for anti-mouse and anti-rabbit)	100 ml (anti-mouse) 100 ml (anti-rabbit)
Incubation Dishes	100 mm x 100 mm x 15 mm plastic dishes with lids (Fisher No. O8-757-11A)	2

Continued on next page

Kit Contents, Continued

WesternBreeze® Chemiluminescent Kit Contents

The components included in the WesternBreeze® Chemiluminescent Immunodetection Kit are listed below. Sufficient reagents are supplied to detect 20 mini-blots.

Item	Description	Amount
Blocker/Diluent (Part A)	Concentrated buffered saline solution containing detergent	80 ml
Blocker/Diluent (Part B)	Concentrated Hammersten casein solution	80 ml
Antibody Wash Solution (16X)	Concentrated buffered saline solution containing detergent	2 x 100 ml
Chemiluminescent Substrate	Ready-to-use solution of CDP-Star® chemiluminescent substrate for alkaline phosphatase.	50 ml
Secondary Antibody Solution	Ready-to-use solution of alkaline phosphatase-conjugated, affinity purified, anti-species IgG (one each for anti-mouse and anti-rabbit)	100 ml (anti-mouse) 100 ml (anti-rabbit)
Incubation Dishes	100 mm x 100 mm x 15 mm plastic dishes with lids (Fisher No. O8-757-11A)	2
Plastic Sheets	5" x 8"	2
Filter Paper Sheets	3-3/8" x 8"	10

Note

- Some reagents in the kit may be provided in excess of the amount needed.
- The NuPAGE® Novex Mini Gels and Invitrolon™ PVDF/Filter membrane sandwiches included in the BioModule™ Western Analysis unit are supplied with individual documentation detailing general use of the product. **For instructions to use these products specifically with the BioModule™ Western Analysis Unit, follow the recommended protocols in this manual.**

Introduction

Overview

Introduction

The BioModule™ Western Analysis Units provide qualified reagents and validated protocols to perform SDS-PAGE, Western transfer, and immunodetection.

The BioModule™ Western Analysis Unit is ideal for confirming the proper expression or accumulation of the protein of interest, identifying a target from a microarray experiment, or validating results from a quantitative PCR (qPCR) or RNA interference (RNAi) experiment.

System Components

The BioModule™ Western Analysis Unit includes:

- The Cell Extraction Buffer and Protease Inhibitor Cocktail for sample preparation from mammalian cells or tissues
- NuPAGE® Novex Bis-Tris Gels, pre-made buffers, and protein standards for high-performance, neutral pH protein electrophoresis
- Pre-cut, pre-assembled, high-quality Invitrolon™ PVDF membrane/filter paper sandwiches for Western transfer of proteins
- WesternBreeze® Immunodetection Kits for ultra-sensitive (femtogram to low picogram-level) detection of proteins without compromising the background

For details on each component, see page 3.

System Overview

To use the BioModule™ Western Analysis Unit, you will:

- Prepare your sample using the Cell Extraction Buffer and Protease Inhibitor
- Perform SDS-PAGE with NuPAGE® Novex Bis-Tris Gels and pre-made buffers
- Transfer the proteins onto the Invitrolon™ PVDF membrane
- Detect the protein of interest using the WesternBreeze® Immunodetection Kits

Advantages

Using the BioModule™ Western Analysis Unit for SDS-PAGE and Western immunodetection offers the following advantages:

- Includes qualified reagents and validated protocols to provide consistent results
- Use of NuPAGE® Novex electrophoresis system for analysis offers best resolution and fast transfer of proteins
- Provides highly sensitive immunodetection with low background using WesternBreeze® Immunodetection Kits

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Purpose of the Manual

This manual provides the following information:

- An overview of the NuPAGE® Protein Electrophoresis System
- Preparing samples and running buffer
- Instructions for performing SDS-PAGE using the XCell *SureLock*™ Mini-Cell
- Western blotting protocol using the XCell™ Blot Module
- Western immunodetection protocol using WesternBreeze® Immunodetection Kits
- Examples of expected results and Troubleshooting

Description of Components

Introduction	Brief description of the components included with the BioModule™ Western Analysis Unit is described in this section.
NuPAGE® Novex Mini Gel System	The NuPAGE® Novex Mini Gel System includes NuPAGE® Novex Bis-Tris Gels and pre-made buffers and is a revolutionary neutral pH, discontinuous SDS-PAGE, pre-cast polyacrylamide mini gel system to perform electrophoresis. The neutral pH 7.0 environment during electrophoresis results in maximum stability of both proteins and gel matrix, providing better band resolution than other gel systems including the Laemmli system. See page 5 for details on the NuPAGE® Electrophoresis System.
Cell Extraction Buffer	The Cell Extraction Buffer is a detergent-based extraction buffer designed for efficient cell lysis and protein solubilization from cultured suspension or adherent mammalian cells, and mammalian tissues. Cell lysates are compatible for use with a variety of downstream applications including electrophoresis and Western blotting.
Protease Inhibitor Cocktail for Mammalian Tissues	The Protease Inhibitor Cocktail for Mammalian Tissues is a mixture of protease inhibitors with a broad specificity for inhibition of a variety of proteases that include serine, cysteine, aspartic, and aminopeptidases. The addition of protease inhibitor cocktail to the lysis buffer during lysis prevents the degradation of proteins due to the proteases present in the cell extracts. This protease inhibitor cocktail is designed for use with mammalian tissues.
WesternBreeze® Immunodetection Kits	The WesternBreeze® Immunodetection Kits contain complete, optimized, ready-to-use or ready-to-dilute reagents for sensitive immunodetection of western blots or dot blots. The kit is used to detect primary antibodies immobilized on nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membranes.
SeeBlue® Plus2 Pre-Stained Standard	The standard allows easy visualization of protein molecular weight ranges during electrophoresis and evaluation of western transfer efficiency. The standard consists of 10 pre-stained protein bands (8 blue and 2 contrasting colors) in the range of 4-250 kDa and is suitable for use with NuPAGE® Novex Gels, Tris-Glycine, or Tricine Gels.

Continued on next page

Description of Components, Continued

MagicMark™ XP Western Protein Standard

The MagicMark™ XP Western Standard allows direct visualization of protein standard bands on a blot without the need for protein modification or special detection reagents. MagicMark™ XP Western Standard proteins are expressed in *E. coli* from a construct containing repetitive units of a fusion protein forming the size variation and an IgG binding site.

The important features of the standard are listed below:

- MagicMark™ XP consists of nine recombinant proteins in the range of 20-220 kDa
- Suitable for Western blotting and molecular weight estimation
- Supplied in a ready-to-use format
- Visualized with alkaline phosphatase or peroxidase conjugated antibody using chromogenic, chemiluminescent, or fluorescent substrates
- Visualized also with SimplyBlue™ SafeStain or other Coomassie® stains on SDS-PAGE gels

The affinity of MagicMark™ XP Standard to various antibodies is listed below:

Species	Affinity of MagicMark™
Human, Horse, Cow	++++
Pig, Rabbit	+++
Goat, Sheep, Hamster, Guinea Pig, Rat, Mouse	++
Chicken	+

Invitrolon™ PVDF

Invitrolon™ PVDF is a high quality, 0.45 µm PVDF membrane particularly suitable for high sensitivity and low background immunoblotting (Western blot analysis) and is ideal for amino acid analysis and protein sequencing of small amounts of proteins (as little as 10 pmoles). Invitrolon™ PVDF is solvent resistant, is physically stronger than nitrocellulose, and compatible with commonly used protein stains and immunodetection methods.

Invitrolon™ PVDF membrane is supplied between two pieces of pre-cut 3 MM filter paper that is used as part of the blot 'sandwich'.

The product specifications are listed below:

Pore Size:	0.45 µm
Dimensions:	8.3 cm x 7.3 cm
Binding Capacity:	Goat IgG: 294 µg/cm^2 BSA: 131 µg/cm^2 Insulin: 85 µg/cm^2
Application:	Western Transfers for Proteins >10 kDa Protein Sequencing Amino Acid Analysis
Re-probe Characteristics:	Yes

NuPAGE® Electrophoresis System

Introduction

General information about the NuPAGE® electrophoresis system is described in this section.

System Components

The NuPAGE® Novex Bis-Tris Gel System consists of:

- NuPAGE® Novex Bis-Tris [Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane-HCl] Mini Gels for separating small to mid-size molecular weight proteins
- NuPAGE® LDS (lithium dodecyl sulfate) Sample Buffer
- NuPAGE® Sample Reducing Agent
- NuPAGE® Antioxidant
- NuPAGE® MES [2-(N-morpholino) ethane sulfonic acid] SDS Running Buffer for NuPAGE® Novex Bis-Tris Mini Gels
- NuPAGE® Transfer Buffer for blotting NuPAGE® Novex Bis-Tris Gels

NuPAGE® Novex Bis-Tris Gel

The NuPAGE® Novex Bis-Tris Gel is a 1.0 mm thick, mini gel (8 cm x 8 cm) used for electrophoresis of protein samples.

The NuPAGE® Novex Bis-Tris Gels are used with NuPAGE® Bis-Tris SDS Buffer System (see below) to produce a discontinuous SDS-PAGE system operating at neutral pH. The neutral pH environment during electrophoresis results in maximum stability of both proteins and gel matrix, providing better band resolution than other gel systems. Visit www.invitrogen.com for types of gels available from Invitrogen.

The size of a NuPAGE® Novex Bis-Tris Gel cassette is 10 cm x 10 cm (gel size is 8 cm x 8 cm). We recommend using the XCell *SureLock*™ Mini-Cell (page 44) for the electrophoresis of NuPAGE® Novex Bis-Tris Gels to obtain optimal and consistent performance.

NuPAGE® Bis-Tris Buffer System

The NuPAGE® Bis-Tris discontinuous buffer system involves three ions:

- Chloride ($^-$) is supplied by the gel buffer and serves as a leading ion due to its high affinity to the anode as compared to other anions in the system. The gel buffer ions are Bis-Tris ($^+$) and Cl^- (pH 6.4).
- MES or MOPS ($^-$) serves as the trailing ion. The running buffer ions are Tris ($^+$), MOPS ($^-$)/MES ($^-$), and dodecylsulfate ($^-$) (pH 7.3-7.7).
- Bis-Tris ($^+$) is the common ion present in the gel buffer and running buffer. The combination of a lower pH gel buffer (pH 6.4) and running buffer (pH 7.3-7.7) results in a significantly lower operating pH of 7.0 during electrophoresis.

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NuPAGE® Electrophoresis System, Continued

Gel Specifications

Gel Matrix:	Acrylamide/Bisacrylamide
Gel Size:	8 cm x 8 cm
Cassette Size:	10 cm x 10 cm
Gel Thickness:	1.0 mm
Cassette Material:	Styrene Copolymer
Sample Well Configuration:	10 well
Maximum Loading Volume:	25 µl (for 1.0 mm thick gel)

The maximum protein load/band is 0.5 µg/band (for Coomassie® stain) while for immunoblotting, you will need to scale the sample load according to the sensitivity of your detection method.

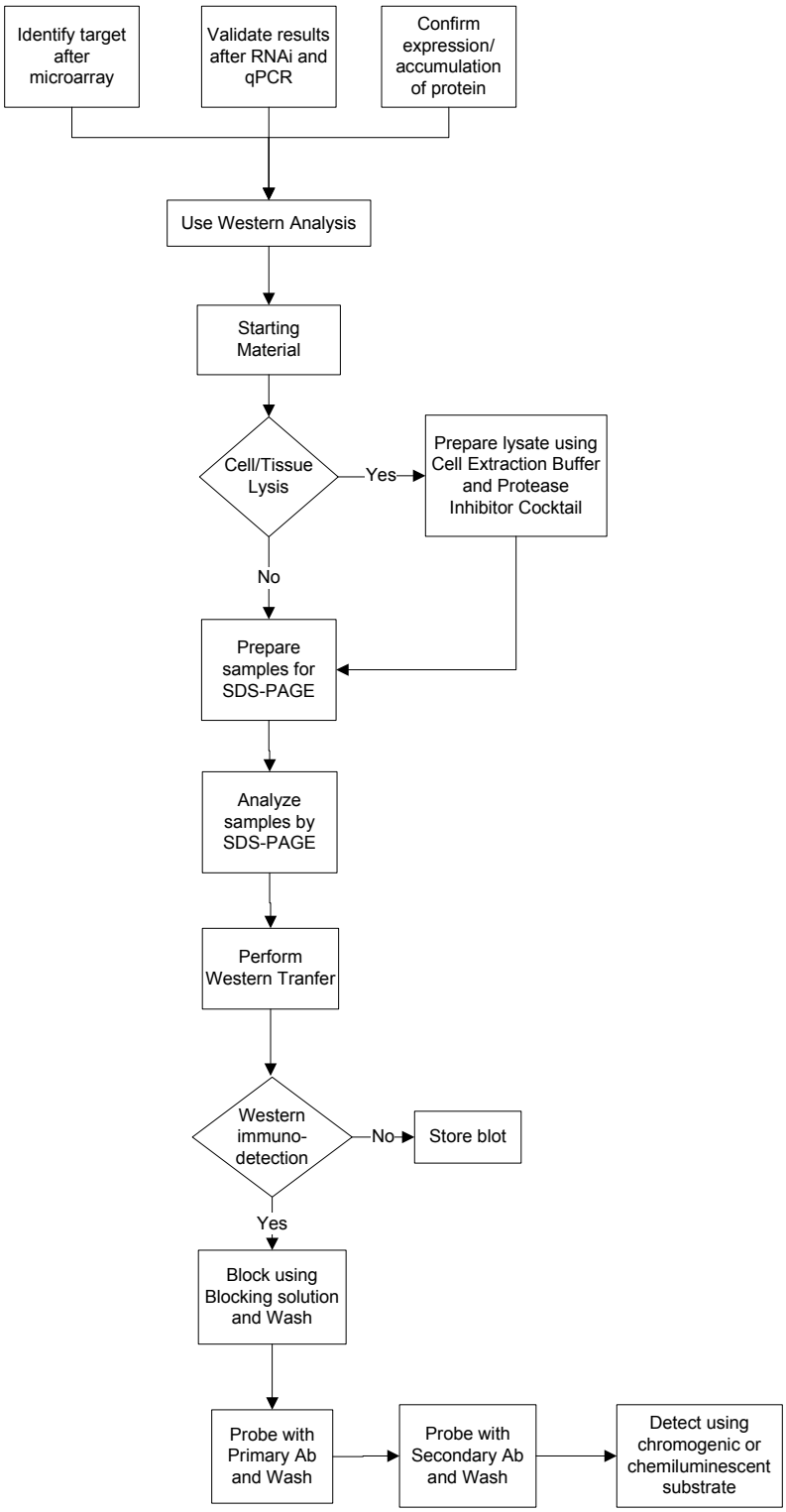
Important

The NuPAGE® Novex Bis-Tris Gels and buffers are designed for denaturing reducing or non-reducing electrophoresis. **Do not use the NuPAGE® Novex Bis-Tris Gels with other buffer systems or under native conditions.**

Experimental Overview

Workflow

The experimental workflow for performing SDS-PAGE, Western transfer, and immunodetection is shown below.



Continued on next page

Experimental Overview, Continued

Materials Needed

Materials supplied with the BioModule™ Western Analysis Unit and User Supplied materials are listed below. Ordering information is on page 44

Supplied in the Module	User Supplied
Lysate Preparation	
• Protease Inhibitors • Cell Extraction Buffer	• Sample of choice • Tissue homogenizer such as the microtube pestle (for tissue samples) • Phosphate Buffered Saline (for cell samples) • Protein quantitation kit or reagents
SDS-PAGE	
• NuPAGE® Novex 4-12% Bis-Tris Gels • NuPAGE® LDS Sample Buffer • NuPAGE® Sample Reducing Agent • NuPAGE® Antioxidant • NuPAGE® MES SDS Running Buffer • SeeBlue® Plus2 Pre-stained Standard • MagicMark™ XP Western Standard	• XCell *SureLock*™ Mini-Cell for electrophoresis of NuPAGE® Gels • Power supply
Western Transfer	
• NuPAGE® Transfer Buffer • NuPAGE® Antioxidant • Invitrolon™ PVDF membrane/filter sandwich	• XCell II™ Blot Module for blotting of NuPAGE® Gels • Power supply • Methanol and deionized water • Stains to stain the blot (optional) • Incubation Tray (optional) • Blotting Roller (optional)
Western Immunodetection	
• WesternBreeze Immunodetection Kit includes the reagents (substrate solution and secondary antibody), buffers (blocking and washing), and plastic dishes (for incubation of the blot)	• Primary Antibody • X-ray film, autoradiography cassette or imaging system for chemiluminescent detection

Methods

Preparing Lysates

Introduction

General guidelines for preparing lysates from cells and tissue are discussed below.

Sample Preparation

Proper sample preparation is key to the success of a Western analysis experiment. Various factors affect the design of a sample preparation protocol.

Due to the large variety of proteins present in different cells and tissues, it is not possible to have a single sample preparation protocol that is suitable for all proteins. Based on the starting material and goal of the experiment, the sample preparation protocol needs to be determined empirically. The sample preparation conditions may also be optimized based on your initial results. If an optimized sample preparation protocol exists in the laboratory for your specific samples, use the optimized protocol.

General guidelines are provided below to prepare samples from various sources, and example procedures are provided on the next page.

Mammalian Cell Samples

A protocol to prepare samples from mammalian cells using the Cell Extraction Buffer with Protease Inhibitor Cocktail is described on the next page. The Cell Extraction Buffer is designed for efficient cell lysis and protein solubilization from cultured suspension or adherent mammalian cells.

Mammalian Tissue Samples

A protocol to prepare samples from mammalian tissue using the Cell Extraction Buffer with Protease Inhibitor Cocktail and tissue homogenizer is described on page 11. The Cell Extraction Buffer is designed for efficient cell lysis and protein solubilization from mammalian tissues.

Samples Already Prepared

If you have samples that are already prepared using a different extraction buffer, proceed directly to preparing samples for SDS-PAGE using the NuPAGE® LDS Sample Buffer (page 13).

Protein Estimation

Use an accurate and sensitive protein estimation method after preparing samples. Choose a protein estimation method that is insensitive to some interfering components present in the extraction buffer. Accurate protein estimation is essential for calculating protein load and performing subsequent detection.

We recommend using the Quant-iT™ Protein Assay Kit (page 44) for easy and sensitive fluorescence-based quantitation of proteins.

We do not recommend estimating protein concentration using UV absorption.

Continued on next page

Preparing Lysates, Continued

Materials Needed

- Sample of interest
- Phosphate buffered saline (PBS) for washing mammalian cells (page 44)
- Sterile microcentrifuge tubes and microcentrifuge
- Cell Extraction Buffer (supplied with the kit); thaw an aliquot quickly at room temperature or on ice and keep on ice until use
- Protease Inhibitor Cocktail (supplied with the kit); thaw an aliquot quickly at room temperature and add the required amount (see below) to the Cell Extraction Buffer and store the cocktail in buffer on ice until use
- Microtube pestle tissue homogenizer (for tissue samples only)

Note

The Protease inhibitor cocktail amount is provided as a starting point for sample preparation in the protocol below. Based on your initial results, you can use the Protease inhibitor cocktail dilution ranging from 1:10 to 1:100.

Preparing Mammalian Cell Samples

A protocol for preparing mammalian cell lysate from 1 x 10^6 cells using 100 µl Cell Extraction Buffer is described below.

1. Grow mammalian cells using standard conditions.
2. After cells have reached the desired density, harvest suspension cells by centrifugation or scrape adherent cells and harvest cells by centrifugation.
3. Wash cells twice with cold PBS to remove any residual media components. Discard the supernatant.
4. During the washing steps, prepare 1 ml **fresh** Cell Extraction Buffer with 1:20 Protease Inhibitor Cocktail as follows:

   To a sterile microcentrifuge tube, add the following:

Cell Extraction Buffer	950 µl
Protease Inhibitor Cocktail	50 µl
Total Volume	1000 µl

5. Immediately return the remaining Cell Extraction Buffer and Protease Inhibitor Cocktail to -20ºC.

   Note: The Cell Extraction Buffer without protease inhibitor is stable for 2-3 weeks at 4ºC, but for long-term storage, aliquot the buffer and store at -20ºC.
6. Resuspend the cell pellet in Cell Extraction Buffer containing the Protease Inhibitor Cocktail prepared in Step 4. The recommended amount of buffer is 10^7-10^8 cells/ml of buffer. A general starting point is resuspending ~1 x 10^6 cells in 100 µl buffer.
7. Incubate the samples on ice for 30 minutes with intermittent vortexing at 10 minute intervals.
8. Centrifuge the samples at 13,000 x g for 10 minutes at 4ºC to remove any particulate material.
9. Transfer and aliquot the supernatant to sterile microcentrifuge tubes and proceed to **Preparing Samples for SDS-PAGE** (page 13) after protein estimation, or store the aliquots at -80ºC.

Continued on next page

Preparing Mammalian Tissue Samples

A protocol for preparing mammalian tissue lysate from 100 mg tissue using 1 ml Cell Extraction Buffer is described below. This protocol is suitable for use with a variety of tissue types; however some optimization may be required for some tissues.

1. Prepare 1 ml **fresh** Cell Extraction Buffer with 1:20 Protease Inhibitor Cocktail as follows:

   To a sterile microcentrifuge tube, add the following:

Cell Extraction Buffer	950 µl
Protease Inhibitor Cocktail	50 µl
Total Volume	1000 µl

2. Immediately return the remaining Cell Extraction Buffer and Protease Inhibitor Cocktail to -20ºC.

   Note: The Cell Extraction Buffer without protease inhibitors is stable for 2-3 weeks at 4ºC, but for long-term storage, aliquot the buffer and store at -20ºC.

3. Cut the tissue into small pieces and place 100 mg tissue in a microcentrifuge tube.
4. Add 1 ml Cell Extraction Buffer containing Protease Inhibitor Cocktail prepared in Step 1 to the tube.
5. Homogenize the tissue using a pestle that fits into the microcentrifuge tube.
6. Incubate the samples on ice for 10 minutes with intermittent vortexing.
7. Centrifuge the lysate at 10,000 x g for 5-10 minutes to remove any particulate material.
8. Transfer and aliquot the supernatant to sterile microcentrifuge tubes and proceed to **Preparing Samples for SDS-PAGE** (page 13) after protein estimation, or store aliquots at -80ºC.

Preparing Samples and Buffers

Introduction

General guidelines for preparing samples and buffers for NuPAGE® Novex Bis-Tris pre-cast gels are discussed below. If you need to prepare lysates from your samples, see page 9.

Positive Control

If you are performing Western Analysis to validate results after RNAi or qPCR experiments, or wish to compare the expression of your protein of interest in various samples, we recommend that you perform detection of β-actin or alpha tubulin using respective antibodies as a positive control for Western analysis and sample loading.

We have validated the BioModule™ Western Analysis Unit using the mouse anti-alpha-tubulin antibody available from Invitrogen (page 44) which reacts with human, mouse, and rat alpha-tubulin.

NuPAGE® LDS Sample Buffer

Use the NuPAGE® LDS Sample Buffer (4X) to prepare samples for denaturing gel electrophoresis with the NuPAGE® Novex Mini Gels.

The NuPAGE® LDS Sample Buffer is formulated to reliably provide complete reduction of the disulfides under mild heating conditions (70ºC for 10 minutes) and eliminate any protein cleavage during sample preparation.

NuPAGE® Sample Reducing Agent

The NuPAGE® Sample Reducing Agent contains 500 mM dithiothreitol (DTT) at a 10X concentration in a ready-to-use, stabilized liquid form (page 44) and is used to reduce samples for electrophoresis.

β-mercaptoethanol can be used with the NuPAGE® gels at a final concentration of 2.5% (v/v). Choice of the reducing agent is a matter of preference and DTT or β-mercaptoethanol can be used. **We recommend adding the reducing agent to the sample within an hour of loading the gel.**

Avoid storing reduced samples for long periods of time even if they are frozen. This will result in the reoxidation of samples during storage and produce inconsistent results.

Important

Do not use the NuPAGE® Antioxidant (see below) as a sample reducing agent. The antioxidant is not efficient in reducing the disulfide bonds. This will result in partially reduced bands with substantial background smearing in the lane.

NuPAGE® Antioxidant

The NuPAGE® Antioxidant (a proprietary reagent) is added to the running buffer in the Upper (cathode) Buffer Chamber only when performing electrophoresis of NuPAGE® Novex Gels under **reducing** conditions. The NuPAGE® Antioxidant migrates with the proteins during electrophoresis preventing the proteins from reoxidizing and maintaining the proteins in a reduced state. The NuPAGE® Antioxidant also protects sensitive amino acids such as methionine and tryptophan from oxidizing.

Continued on next page

Preparing Samples and Buffers, Continued

Materials Needed

You will need the following items:

- Cell or tissue lysate
- Sterile microcentrifuge tubes
- Heat block set to 70ºC
- NuPAGE® LDS Sample Buffer, NuPAGE® Sample Reducing Agent; NuPAGE® MES SDS Running Buffer; supplied with the kit
- Microcentrifuge

Preparing Samples for SDS-PAGE

Instructions are provided below to prepare reduced or non-reduced samples for denaturing gel electrophoresis using the NuPAGE® Novex Bis-Tris Gels.

For reduced sample, add the reducing agent immediately prior to electrophoresis to obtain the best results.

Prepare your samples in a total volume of 10 µl as described below. If you need to prepare samples in a volume of 5-25 µl, adjust the volume accordingly.

1. To a sterile microcentrifuge tube, add the following:

Reagent	Reduced Sample	Non-reduced Sample
Sample	x µl	x µl
NuPAGE® LDS Sample Buffer (4X)	2.5 µl	2.5 µl
NuPAGE® Reducing Agent (10X)	1 µl	--
Deionized Water	to 6.5 µl	to 7.5 µl
Total Volume	10 µl	10 µl

2. Heat samples at 70ºC for 10 minutes. Immediately load the samples on the gel, next page.

Preparing 1X Running Buffer for SDS-PAGE

You will need 1000 ml 1X NuPAGE® MES SDS Running Buffer for electrophoresis with the XCell *SureLock*™ Mini-Cell.

1. Prepare 1000 ml 1X NuPAGE® MES SDS Running Buffer using NuPAGE® MES SDS Running Buffer (20X) as follows:

NuPAGE® MES SDS Running Buffer (20X)	50 ml
Deionized Water	950 ml
Total Volume	1000 ml

2. Mix thoroughly and set aside 800 ml of the 1X NuPAGE® MES SDS Running Buffer for use in the Lower (Outer) Buffer Chamber of the XCell *SureLock*™ Mini-Cell.
3. **For reduced samples:** Immediately, prior to electrophoresis, add 500 µl NuPAGE® Antioxidant to 200 ml 1X NuPAGE® MES SDS Running Buffer from Step 1 for use in the Upper Buffer Chamber of the XCell *SureLock*™ Mini-Cell. Mix thoroughly.

Performing SDS-PAGE

Introduction

Instructions to perform SDS-PAGE under denaturing conditions using the XCell *SureLock*™ Mini-Cell are described in this section.

Note

The NuPAGE® Novex Mini Gels do not contain SDS. However, the mini gels are designed for performing denaturing gel electrophoresis. **Do not** use the NuPAGE® Bis-Tris Gels with NuPAGE® MES Running Buffer without SDS for native gel electrophoresis. This buffer system may generate excessive heat resulting in poor band resolution. The protein of interest may not migrate very well in a neutral pH environment if it is not charged.

Running Reduced and Non-Reduced Samples

For optimal results, we do not recommend running reduced and non-reduced samples on the same gel.

If you do choose to run reduced and non-reduced samples on the same gel, follow these guidelines:

- Do not run reduced and non-reduced samples in adjacent lanes. The reducing agent may have a carry-over effect on the non-reduced samples if they are in close proximity.
- If you are running reduced and non-reduced samples on the same gel, omit the NuPAGE® Antioxidant in the running buffer. The antioxidant will have a deleterious effect on the non-reduced samples. The bands will be sharper on NuPAGE® Gels relative to other gel systems, even without the use of the antioxidant.

Protein Standards

The SeeBlue® Plus2 Pre-Stained Standard and MagicMark™ XP Western Standard are supplied ready-to-use. There is no need to heat or add reducing agent. Use the SeeBlue® Plus2 Pre-Stained Standard to monitor electrophoresis and transfer efficiency, and use the MagicMark™ XP Western Standard to visualize protein standard bands on a blot.

The protein standards can be run on separate lanes or can be pre-mixed together and run on a single lane.

Use loading volumes listed on page 16 to obtain the best results.

Continued on next page

Performing SDS-PAGE, Continued

Experimental Outline

1. Prepare gel cassettes by removing the tape at the bottom and combs followed by rinsing the sample wells.
2. Assemble the XCell *SureLock*™ Mini-Cell with the cassettes.
3. Add 1X Running Buffer to the Upper Buffer Chamber.
4. Load protein samples and standards into the wells.
5. Add 1X Running Buffer to the Lower Buffer Chamber, place the lid on the mini-cell, and connect the mini-cell to a power supply.
6. Perform electrophoresis.

Materials Needed

You will need the following items:

- XCell *SureLock*™ Mini-Cell (page 44)
- Power Supply (page 44)
- Protein Sample (prepared as described on page 13)
- Protein Standards (supplied with the kit)
- 1X Running Buffer (prepared as described on page 13)

Gels are individually packaged in clear pouches with 10 ml of Packaging Buffer. The Packaging Buffer contains low levels of residual acrylamide monomer and 0.02% sodium azide.

To avoid contamination from possible residual acrylamide, wear gloves when handling gels.

Warning: This product contains a chemical (acrylamide) known to the state of California to cause cancer. To obtain a MSDS, see page 41

Preparing Gel Cassettes

Wear protective gloves and safety glasses when handling gels.

1. Cut open the gel cassette pouch and drain away the gel packaging buffer.
2. Remove the gel cassette from the pouch and rinse with deionized water.
3. Peel off the tape covering the slot on the back of the gel cassette.
4. In one fluid motion, pull the comb out of the cassette.
5. Use a pipette to gently wash the cassette wells with 1X Running Buffer. Invert the gel and shake to remove buffer. Repeat twice. Fill the sample wells with1X Running Buffer. Be sure to displace all air bubbles from the cassette wells, as they will affect sample running.

Note: Always handle the cassette by its edges only.

Continued on next page

Procedure using XCell *SureLock*™ Mini-Cell

XCell *SureLock*™ Mini-Cell requires 200 ml for the Upper Buffer Chamber and 600 ml for the Lower Buffer Chamber.

1. Orient the two gels in the Mini-Cell such that the notched "well" side of the cassette faces inwards toward the Buffer Core. Seat the Buffer core with gels on the bottom of the Mini-Cell and lock into place with the Gel Tension Wedge. Refer to the XCell *SureLock*™ Mini-Cell manual for detailed instructions.

   Note: If you are using only one gel, the plastic Buffer Dam replaces the second gel.

2. Fill the Upper Buffer Chamber with a small amount of the running buffer to check for tightness of seal. If you detect a leak from Upper to the Lower Buffer Chamber, discard the buffer, reseal the chamber, and refill.

3. Once the seal is tight, fill the Upper Buffer Chamber (inner) with 200 ml 1X Running Buffer. Slowly load the buffer into the Upper Buffer Chamber to avoid a lot of bubble formation as bubbles make the sample loading more difficult. The buffer level must exceed the level of the wells.

   Note: If you are running reduced samples, fill the Upper Buffer Chamber with 200 ml 1X Running Buffer containing the NuPAGE® Antioxidant (page 13).

4. Using a pipette equipped with a sample loading tip, underlay the following samples into the gel wells.

   - Load 10-25 µl sample at the desired protein concentration onto each well of the gel.
   - Pre-mix 10 µl MagicMark™ XP Western Standard with 5 µl SeeBlue® Plus2 Pre-stained Standard. Load 15 µl of the protein standard mixture onto a well to allow you to monitor the electrophoresis run and visualize protein standard bands on the blot after protein transfer

     OR

   - Load 10 µl MagicMark™ XP Western Standard and 10 µl SeeBlue® Plus2 Pre-stained Standard onto two separate wells.

   Note: To obtain the best results and promote a uniform running of the stacking front, load sample buffer in all the wells, whether or not they contain samples.

5. Fill the Lower Buffer Chamber (anode) by pouring 600 ml 1X Running Buffer through the gap between the Gel Tension Wedge and the back of the Lower Buffer Chamber.

⚠ Do not handle the lid if the cables are plugged into the power supply.

6. Place the lid on the assembled XCell *SureLock*™ Mini-Cell. The lid firmly seats if the (-) and (+) electrodes are properly aligned. If the lid is not properly seated, no power goes through the Mini-Cell.

7. With the power **off**, connect the electrode cords to power supply {red to (+) jack, black to (-) jack}.

⚠ Ensure the power is off before connecting the Mini-Cell to the power supply.

8. Turn on the power. See next page for electrophoresis conditions.

Continued on next page

Electrophoresis Conditions

Once you have assembled the XCell4 *SureLock*™ Midi-Cell and loaded your samples, you are ready to perform electrophoresis.

You will need a power supply designed for electrophoresis (page 44).

The electrophoresis conditions for NuPAGE® Novex Bis-Tris Gels using the XCell *SureLock*™ Mini-Cell are listed below.

Gel Type	Voltage	Expected Current*	Run Time
NuPAGE® Bis-Tris SDS-PAGE (denaturing, reducing or non-reducing) with MES Running Buffer	200 V Constant	Start: 110-125 mA End: 70-80 mA	35 minutes

*Current readings are per gel

At the end of the run, turn off the power, and disconnect cables from the power supply. Proceed immediately to **Disassembling the XCell *SureLock*™ Mini-Cell**, below.

Disassembling the XCell *SureLock*™ Mini-Cell

Refer to the instructions below to disassemble the XCell *SureLock*™ Mini-Cell.

Be sure the cables are disconnected from the power supply.

1. Remove the lid. Discard the buffer, and unlock the Gel Tension Wedge.
2. Remove the Buffer Cores with the gel cassettes from the Lower Buffer Chamber while holding the cassettes against the cores.
3. Remove the gel cassettes from the Buffer Cores and lay the gel cassettes (well side up) on a flat surface, such as the benchtop. Allow one edge to hang ~1 cm over the side of the benchtop.
4. Carefully insert the Gel Knife's beveled edge into the narrow gap between the two plates of the cassette.

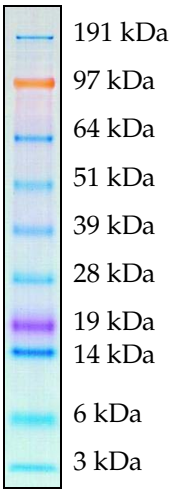
   Caution: Use caution while inserting the Gel Knife between the two plates to avoid excessive pressure towards the gel.
5. Push up and down gently on the knife's handle to separate the plates. You will hear a cracking sound indicating that you have broken the bonds that hold the plates together. Repeat on each side of the cassette until the plates are completely separated.
6. Upon opening the cassette, the gel may adhere to either side. Remove and discard the plate without the gel, allowing the gel to remain on the other plate.
7. Proceed to the **Western Blotting Protocol** (page 19). See next page for an example of expected results after SDS-PAGE.

Continued on next page

Example of Expected Results

An example of results obtained after SDS-PAGE of SeeBlue® Plus2 Pre-Stained Standard is shown below.

The SeeBlue® Plus2 Pre-Stained Standard was electrophoresed on a NuPAGE® Novex 4-12% Bis-Tris Gel using the NuPAGE® MES SDS Running Buffer. The apparent molecular weights of the protein bands in SeeBlue® Plus2 Pre-Stained Standard are listed below (see below).



Western Blotting Protocol

Introduction

The blotting protocol described below is suitable for majority of protein blotting applications using the XCell II™ Blot Module. However, some optimization may be necessary to obtain the best results (page 26).

XCell II™ Blot Module

The XCell II™ Blot Module is a simple apparatus designed for blotting of mini-gels and is easily inserted into the XCell *SureLock*™ Mini-Cell in place of the gel/buffer core assembly. The module has rails to guide the unit into the mini-cell. The XCell II™ Blot Module is a semi-wet transfer unit and can be used to perform western, Southern, or northern transfer of two mini-gels using only 200 ml of transfer buffer. An efficient transfer is obtained, as the resistance is constant across the blotting electrodes producing uniform field strength.

Wear gloves at all times during the entire blotting procedure to prevent contamination of gels and membranes and to avoid exposure to irritants commonly used in blotting procedures.

Do not touch the membrane or gel with bare hands. This may contaminate the gel or membrane and interfere with further analysis.

Materials Needed

You will need the following items:

- Invitrolon™ PVDF membrane/filter paper sandwiches (supplied with the kit)
- Methanol
- Deionized water
- Transfer buffer (see next page)
- XCell II™ Blot Module (page 44)
- Power Supply (page 44)
- *Optional:* Incubation Tray and Blotting Roller (page 44)

Experimental Outline

1. Prepare the transfer buffer, membranes, and blotting pads.
2. Assemble the XCell II™ Blot Module with the gel and membranes and insert the blot module into the XCell *SureLock*™ Mini-Cell.
3. Add Transfer Buffer to the blot module and deionized water to the outer chamber.
4. Place the lid on the unit and connect the unit to a power supply.
5. Perform western transfer.

Continued on next page

Western Blotting Protocol, Continued

Preparing Transfer Buffer

Prepare 1000 ml of 1X NuPAGE® Transfer Buffer using the NuPAGE® Transfer Buffer (20X) as follows:

Reagents	One Gel	Two Gels
NuPAGE® Transfer Buffer (20X)	50 ml	50 ml
Methanol*	100 ml	200 ml
Deionized Water	850 ml	750 ml
Total Volume	1000 ml	1000 ml

*NuPAGE® Transfer Buffer with 10% methanol provides optimal transfer of a single gel in the blot module and for transferring 2 gels in the blot module, increase the methanol content to 20% to ensure efficient transfer of both gels.

To perform transfer of **reduced samples**, add 1 ml NuPAGE® Antioxidant to 1 L 1X NuPAGE® Transfer Buffer prepared as described above.

Preparing Blotting Pads

Use ~ 700 ml 1X Transfer Buffer to soak the blotting pads until saturated. Remove air bubbles by squeezing the blotting pads while they are submerged in buffer. Removing air bubbles is essential as they can block the transfer of biomolecules.

Preparing Membranes

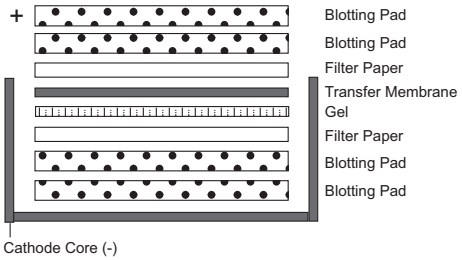
- **PVDF membrane**: Pre-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol. Briefly rinse in deionized water and then place the membrane in a shallow dish containing 50-100 ml transfer buffer for several minutes.
- **Filter paper**: Soak briefly in transfer buffer immediately before using.
- **Gel**: Use the gel immediately following the run (page 17). **Do not soak the gel in transfer buffer**.

Continued on next page

Transferring One Gel

Instructions are provided below for transferring one gel. If you need to transfer two gels at a time, refer to page 23.

1. Place a piece of pre-soaked filter paper on top of the gel and remove air bubbles by rolling a Blotting Roller or glass pipette over the surface. Turn the plate over so the gel and filter paper are facing downwards over a gloved hand.
2. Wet the surface of the gel with the transfer buffer and place pre-soaked transfer membrane on the gel. Remove air bubbles by rolling a Blotting Roller or glass pipette over the membrane surface.
3. Place the pre-soaked filter paper on top of the transfer membrane. Remove any trapped air bubbles using the Blotting Roller.
4. Place 2 soaked blotting pads into the cathode (-) core of the blot module. The cathode core is the deeper of the 2 cores and the corresponding electrode plate is a darker shade of gray. Carefully pick up the gel membrane assembly with your gloved hand and place on the pad in the same sequence, such that the gel is closest to the cathode plate (see figure below).



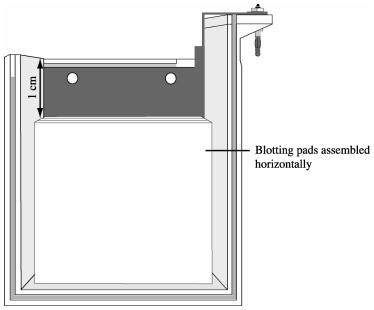
5. Add enough pre-soaked blotting pads to rise 0.5 cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The gel/membrane sandwich should be held securely between the two halves of the blot module ensuring complete contact of all components.

   Note: To ensure a snug fit, use an additional pad since pads lose their resiliency after many uses. Replace pads when they begin to lose resiliency and are discolored.

Continued on next page

Transferring One Gel, continued

6. Position the gel membrane sandwich and blotting pads in the cathode core of the XCell II™ Blot Module to fit horizontally across the bottom of the unit. There should be a gap of ~ 1 cm at the top of the electrodes when the pads and assembly are in place (see figure below).



7. Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module fits into the unit in only one way, such that the (+) sign is seen in the upper left hand corner of the blot module. The inverted gold post on the right hand side of the blot module fits into the hole next to the upright gold post on the right side of the lower buffer chamber.
8. Place the gel tension wedge such that the vertical face of the wedge is against the blot module. Push the lever forward to lock it into place.

   Note: When properly placed, the rear wedge will not be flush with the top of the lower buffer chamber. There will be a gap between the rear wedge and lower chamber.
9. Fill the blot module with 1X Transfer Buffer until the gel/membrane sandwich is covered in buffer. Do not fill all the way to the top, as this will generate extra conductivity and heat.
10. Fill the Outer Buffer Chamber with ~650 ml deionized water by pouring in the gap between the front of the blot module and front of the Lower Buffer Chamber. The water level should reach approximately 2 cm from the top of the Lower Buffer Chamber. This serves to dissipate heat during the run.

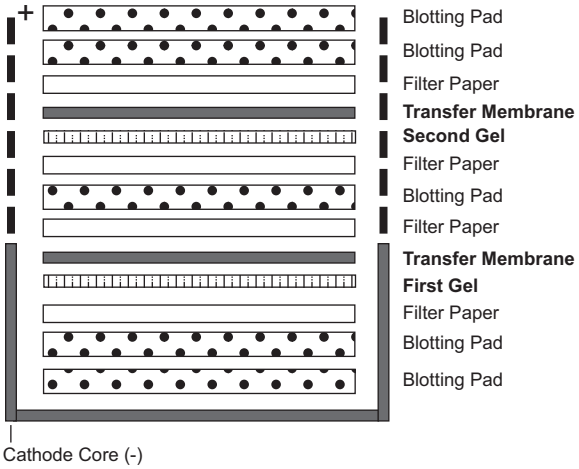
    Note: If you accidentally fill the Outer Buffer Chamber with the transfer buffer, it will not adversely affect the transfer. The liquid in the Outer Buffer Chamber serves as a coolant. We recommend adding deionized water to the Outer Buffer Chamber to avoid any exposure of the mini-cell to methanol as the mini-cell is susceptible to methanol.
11. Place the lid on top of the unit.
12. With the power turned off, plug the red and black leads into the power supply. Perform transfer using the **Transfer Conditions** on the next page.

Continued on next page

Transferring Two Gels

Instructions are provided below for transferring two gels.

1. Remove the gels after electrophoresis as described on page 17.
2. Assemble the gel/membrane sandwich (as described on page 21) twice to make two gel/membrane sandwiches.
3. Place two pre-soaked pads on cathode core of the blot module. Place the first gel/membrane sandwich on pads in the correct orientation, so the gel is closest to the cathode plate (see figure below).



4. Add another pre-soaked blotting pad on top of the first membrane assembly.
5. Position the second gel/membrane sandwich on top of blotting pad in the correct orientation so that the gel is closest to the cathode side.
6. Proceed with Steps 5-12 as described in **Transferring One Gel** (page 21).

Transfer Conditions

The transfer conditions for NuPAGE® Novex Bis-Tris Gels using 1X NuPAGE® Transfer Buffer with 10% methanol for transfer of one gel (with or without NuPAGE® Antioxidant) onto a PVDF membrane are:

30 V constant for 1 hour
Start Current: 170 mA
End Current: 110 mA

Note: The expected current listed in the table below is for transferring one gel. If you are transferring two gels in the blot module, the expected current will double.

Overnight Blotting

For overnight blotting, perform transfer in the cold room with low power to prevent overheating. Transfer at constant voltage of 10-15 V overnight. Depending on the transfer efficiency, adjust the transfer conditions accordingly.

Continued on next page

Semi-Dry Blotting

The NuPAGE® Novex Bis-Tris Gels do not transfer efficiently using a semi-dry transfer cell as compared to blotting with XCell II™ Blot Module. If you decide to use semi-dry blotting for NuPAGE® Novex Bis-Tris Gels, use the protocol provided below to ensure efficient transfer.

1. Prepare 100 ml 2X NuPAGE® Transfer Buffer from the supplied 20X NuPAGE® Transfer Buffer as follows:

NuPAGE® Transfer Buffer (20X)	10.ml
NuPAGE® Antioxidant (reduced sample)	0.1 ml
Methanol	10 ml
Deionized Water	79.9 ml
Total Volume	100 ml

   If you are blotting large proteins, see the **Note** below.
2. Soak the filter paper and transfer membrane in the transfer buffer. For pre-cut membrane/filter sandwiches, use three filter papers (0.4 mm/filter in thickness) on each side of the gel or membrane.
3. Assemble the gel/membrane/filter paper sandwich on top of the anode plate as follows:
   Filter Paper
   Filter Paper
   Filter Paper
   Membrane
   Gel
   Filter Paper
   Filter Paper
   Filter Paper
4. Perform the transfer at 15 V constant for 15 minutes if you are using the Bio-Rad Trans-Blot Semi-Dry Cell. For any other semi-dry transfer cell, follow the manufacturer's recommendations.

Note: For transfer of large proteins (>100 kDa), pre-equilibrate the gel in 2X NuPAGE® Transfer Buffer (without methanol) containing 0.02-0.04% SDS for 10 minutes before assembling the sandwich.

Cleaning the Blot Module

Rinse the blot module with distilled water after use. To clean any residual build-up in the blot module, apply 50% nitric acid in deionized water to areas inside the blot module until residual build-up is removed. Do not submerge the blot module or soak overnight in nitric acid. Use gloves when preparing the nitric acid solution. Once the build-up is removed, rinse the module at least three times in deionized water.

Continued on next page

Post-Transfer Analysis

After the transfer, you may proceed to immunodetection, store the membrane for future use, or stain the membrane.

- For immunodetection of proteins, proceed to the WesternBreeze® Chromogenic or Chemiluminescent Immunodetection protocol (page 28).
- For storing PVDF membranes, air dry the membrane and store the membrane in a air-tight plastic bag at room temperature, 4°C, or -80°C. When you are ready to use the membrane, re-wet the membrane with methanol for a few seconds, followed by thorough rinsing of the membrane with deionized water to remove methanol.
- For staining the membranes after blotting, you may use:
  - 0.1% Coomassie® Blue R-250 in 50% methanol. Do not use Novex® Colloidal Blue Staining Kit for staining of membranes, as the background is high.
  - 20 ml SimplyBlue™ SafeStain (page 44) with dry PVDF membranes and incubate for 1-2 minutes. Wash the membrane three times with 20 ml of deionized water for 1 minute. To avoid high background, do not use SimplyBlue™ SafeStain on **wet** PVDF membranes.
  - SYPRO® Ruby Blot Stain (page 44)
  - 0.1% Ponceau S in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or 10% acetic acid to obtain permanent staining, and air dry.

If you do not detect any proteins on the membrane after immunodetection or staining, refer to the **Troubleshooting** section on page 36.

Testing Transfer Efficiency

The protein bands of SeeBlue® Plus2 Pre-stained Standard are covalently bound to a synthetic dye enabling the visualization of the protein standards during electrophoresis and after the transfer. The transfer efficiency is good, if most of the standard bands have transferred to the membrane (see page 27 for an example of expected results). Note that high molecular weight standards do not always transfer completely and this is not indicative of incomplete transfer.

If none of the standards or only a few have transferred to the membrane, you may have to optimize the transfer conditions. Refer to **Optimizing Blotting Parameters** on the next page and the **Troubleshooting** section on page 36.

Continued on next page

Optimizing Blotting Parameters

When using the XCell II™ Blot Module, most proteins transfer efficiently using the protocol on page 21.

Based on specific properties of a protein or a set of proteins, some optimization of the blotting protocol may be necessary as described below:

Field Strength

A higher field strength (volts/cm) may help larger proteins to transfer, but may also cause smaller proteins to pass through the membrane without binding. Our recommended condition for most proteins is 30 Volts for 60 minutes. You may make minor adjustments (± 5-10V) accordingly, when necessary. See **Transfer Conditions**. (page 23).

Alcohol in Transfer Buffer

Decreasing or eliminating alcohol may improve the transfer of some proteins, especially large proteins.

For blotting 2 NuPAGE® Novex Gels, 20% methanol is added to the transfer buffer. This is balanced by the residual SDS in the gel from the running buffer. Keep this in mind when adjusting the methanol content in transfer buffer.

SDS in Transfer Buffer

Adding 0.01-0.02% SDS to the transfer buffer facilitates the transfer of proteins, especially large proteins, but may reduce binding of proteins.

In the blotting protocol on page 21, the gel is not incubated in the transfer buffer, leaving residual SDS in the gel. This is balanced by the 10-20% methanol added to the transfer buffer. Keep this in mind when adjusting the SDS content in the transfer buffer.

Transfer Time

Increasing the transfer time to two hours improves transfer of most proteins, but may cause the smaller proteins to pass through the membrane. Transfer time usually has little influence on the detection of proteins that remain bound to the membrane. Transfer times longer than 2 hours at the recommended power settings do not greatly improve transfer of proteins that have failed to transfer completely in 2 hours. This may be due to the exhaustion of the buffer or partial fixation of the protein in the gel as a result of the removal of SDS or a conformational change in the proteins during the transfer interval.

We recommend using two membranes in tandem during initial blotting to closely monitor the protein transfer and then perform the same visualization technique on both membranes. Monitor whether the primary membrane located next to the gel retains majority of the sample. If the sample is detected on the membrane placed closer to the anode (further away from the gel), reduce the rate of transfer by lowering the field strength, allowing more time for protein capture on the primary membrane. Optimize the blotting protocol accordingly using the guidelines described above.

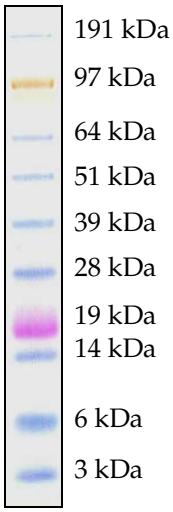
Continued on next page

Example of Expected Results

An example of results obtained after SDS-PAGE and Western transfer of SeeBlue® Plus2 Pre-Stained Standard is shown below.

The SeeBlue® Plus2 Pre-Stained Standard was electrophoresed on a NuPAGE® Novex 4-12% Bis-Tris Gel using the NuPAGE® MES SDS Running Buffer. The standards were blotted onto Invitrolon™ PVDF membrane using the protocol described in this manual. The figure below shows the standard bands after transfer onto PVDF membrane.

The SeeBlue® Plus2 Pre-Stained Standard allows you to quickly evaluate western transfer efficiency. The transfer efficiency is good, if most of the standard bands have transferred to the membrane. If none of the standards or only a few have transferred to the membrane, refer to optimizing the transfer conditions (page 26) and the **Troubleshooting** section on page 36.



WesternBreeze® Chemiluminescent Immunodetection Protocol

Introduction

Instructions for immunodetection using the WesternBreeze® Chemiluminescent Kit are described in this section.

To perform immunodetection using the WesternBreeze® Chromogenic Kit, see page 32.

Safety Issues

There are no hazardous chemicals in the WesternBreeze® Chemiluminescent Immunodetection Kit. However, the system contains dilute solutions of irritating chemicals. We recommend wearing gloves, safety glasses, and a laboratory coat should when using the WesternBreeze® Chemiluminescent Immunodetection Kit.

Important Guidelines

To obtain the best results with WesternBreeze® Chemiluminescent Immunodetection Kit:

- Use a single, clean dish supplied with the kit for each blot.
- Avoid touching the working surface of the membrane, even with gloves.
- Use pure water, free from alkaline phosphatase activity. Autoclave stored water to remove alkaline phosphatase activity. Freshly ultra-filtered water is preferred.
- Avoid cross-contamination of system solutions especially with the alkaline phosphatase substrate solution.
- Perform all washing, blocking, and incubating steps on a rotary shaker platform rotating at 1 revolution/second.
- Work quickly when changing solutions as PVDF membranes dry quickly. If the membrane dries, re-wet the membrane with methanol and rinse with water before proceeding.
- Add solutions to the trays slowly, at the membrane edge, to avoid bubbles forming under the membrane. Decant from the same corner of the dish to ensure complete removal of previous solutions.

Materials Needed

You will need the following items:

- Blotted membranes containing applied antigen or primary antibody samples
- WesternBreeze® Chemiluminescent reagents (included with the kit)
- Primary antibody to detect applied antigen, if appropriate
- Purified water autoclaved, sterile or ultra filtered to remove alkaline phosphatase activity from all solutions used in the procedure
- Clean flasks for preparing solutions
- Forceps for manipulating blotted membranes.
- Orbital shaker platform
- X-ray film, autoradiography cassette and appropriate imaging system

Continued on next page

WesternBreeze® Chemiluminescent Immunodetection Protocol, Continued

Experimental Outline

1. Prepare the solutions for immunodetection using the reagents supplied in the kit.
2. Perform the blocking step to block any non-specific binding.
3. Add diluted primary antibody followed by washing and addition of diluted secondary antibody.
4. Develop the blot using the chemiluminescent substrate and expose the blot to an X-ray film to view the bands.

Preparing Solutions

For western blots from NuPAGE® gels, prepare the solutions for PVDF membranes as described in the table below.

Solution	For PVDF Membrane	
Blocking Solution	Ultra filtered Water	5 ml
	Blocker/Diluent (Part A)	2 ml
	Blocker/Diluent (Part B)	3 ml
	Total Volume	10 ml
Primary Antibody Diluent	Ultra filtered Water	7 ml
	Blocker/Diluent (Part A)	2 ml
	Blocker/Diluent (Part B)	1 ml
	Total Volume	10 ml
	Dilute your primary antibody into this diluent according to the manufacturer's recommendations. Typically, commercial primary antibody preparations are diluted 1:1000 to 1:5000 to a concentration of about 1-0.2 µg/ml.	
Antibody Wash	Ultra filtered Water	150 ml
	Antibody Wash Solution (16X)	10 ml
	Total Volume	160 ml
Chemiluminescent Substrate	Use the CDP-Star® directly from the bottle. **Do not** add the Nitro-Block-II enhancing reagent.	
	Total Volume	2.5 ml

Continued on next page

WesternBreeze® Chemiluminescent Immunodetection Protocol, Continued

Protocol

1. After electrophoresis, wash the membrane twice with deionized water for 5 minutes each time.
2. Place the membrane in 10 ml Blocking Solution in a dish provided in the kit. Incubate for 30 minutes on a rotary shaker set at 1 revolution/second. Decant the Blocking Solution.
3. Rinse the membrane with 20 ml deionized water for 5 minutes, then decant. Repeat once.
4. Incubate the membrane with 10 ml Primary Antibody Solution for 1 hour, then decant.
5. Wash membrane for 5 minutes with 20 ml 1X Antibody Wash, then decant. Repeat 3 times.
6. Incubate the membrane in 10 ml Secondary Antibody Solution for 30 minutes, then decant.
7. Wash the membrane for 5 minutes with 20 ml 1X Antibody Wash, then decant. Repeat 3 times.
8. Rinse the membrane with 20 ml deionized water for 2 minutes, then decant. Repeat twice.
9. Place the membrane on a sheet of transparency plastic. Do not allow the membrane to dry out.
10. With a clean pipette, evenly apply 2.5 ml Chemiluminescent Substrate solution to the membrane surface without touching the membrane surface. Let the reaction develop for 5 minutes.
11. Blot any excess Chemiluminescent Substrate solution from the membrane surface with the filter paper in the kit. Do not allow the membrane to dry out.
12. Cover the membrane with another clean piece of transparency plastic to prepare a membrane sandwich for luminography. Expose an X-ray film (we recommend Kodak X-OMAT AR films) to the membrane sandwich for 1 second to several minutes.

See page 35 for an example of expected results.

The alkaline phosphatase-activated CDP-Star® produces a maximum light emission wavelength at 466 nm to 461 nm, depending on the membrane environment of the reaction.

Continued on next page

WesternBreeze® Chemiluminescent Immunodetection Protocol, Continued

Reprobing the Membrane

If you wish to detect another protein (must be of a size that is not very close to the previous protein that was detected) on the same blot, you can reprobe the same blot using another primary antibody using the following protocol:

1. Re-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol.
2. Wash the membrane twice with deionized water for 5 minutes each time.
3. Resume the immunodetection protocol starting from the blocking step using a different primary antibody.

Stripping and Reprobing the Membrane

If you wish to detect another protein that is in the same size range as the previous protein detected on the same blot, you need to strip the blot to remove the bound primary antibody and then reprobe using another primary antibody as described below.

1. Re-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol.
2. Wash the membrane twice with deionized water for 5 minutes each time.
3. Wash the membrane twice in 1X Antibody Wash Solution for 2 minutes each time.
4. Incubate the membrane in Stripping Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) with shaking at 70ºC water bath for 30 minutes.

   Note: You can use commercially available buffer for stripping the blot.
5. Decant the Stripping Buffer and wash the membrane twice in 1X Antibody Wash solution for 10 minutes each time.
6. Resume the detection protocol starting from the blocking step using another primary antibody.

Performing Chromogenic Detection After Chemiluminescent Detection

If you wish to perform chromogenic detection after chemiluminescent detection to resolve bands that are very close to each other and appear as a thick, single band after chemiluminescent detection, or need to maintain a permanent record of your blot, you can perform chromogenic detection after chemiluminescent detection as follows:

1. Re-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol.
2. Wash the membrane twice with deionized water for 1-2 minutes each time.
3. Add the chromogenic substrate and proceed with detection as described on page 34.

WesternBreeze® Chromogenic Immunodetection Protocol

Introduction

Instructions for immunoblotting using the WesternBreeze® Chromogenic Kit are described in this section.

To perform immunodetection using the WesternBreeze® Chemiluminescent Kit, see page 28.

Safety Issues

The WesternBreeze® Chromogenic Immunodetection Kit contains a solution of 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) and nitro blue tetrazolium (NBT) which is a possible carcinogen and dilute solutions of irritating chemicals. We recommend wearing gloves, safety glasses, and a laboratory coat when using the WesternBreeze® Chromogenic Immunodetection Kit.

Important Guidelines

To obtain the best results with WesternBreeze® Chromogenic Immunodetection Kit:

- Use a single, clean dish supplied with the kit for each blot.
- Avoid touching the working surface of the membrane, even with gloves.
- Use pure water, free from alkaline phosphatase activity. Autoclave stored water to remove alkaline phosphatase activity. Freshly ultra-filtered water is preferred.
- Avoid cross-contamination of system solutions especially with the alkaline phosphatase substrate solution.
- Perform all washing, blocking, and incubating steps on a rotary shaker platform rotating at 1 revolution/second.
- Work quickly when changing solutions as PVDF membranes dry quickly. If the membrane dries, re-wet the membrane with methanol and rinse with water before proceeding.
- Add solutions to the trays slowly, at the membrane edge, to avoid bubbles forming under the membrane. Decant from the same corner of the dish to ensure complete removal of previous solutions.

Materials Needed

You will need the following items:

- Blotted membranes containing applied antigen or primary antibody samples
- WesternBreeze® Chromogenic reagents (included with the kit)
- Primary antibody to detect applied antigen, if appropriate
- Purified water autoclaved, sterile, or ultra filtered to remove alkaline phosphatase activity from all solutions used in the procedure
- Clean flasks for preparing solutions
- Forceps for manipulating blotted membranes
- Orbital shaker platform

Continued on next page

WesternBreeze® Chromogenic Immunodetection Protocol,
Continued

Experimental Outline

1. Prepare the solutions for immunodetection using the reagents supplied in the kit.
2. Perform the blocking step to block any non-specific binding.
3. Add diluted primary antibody followed by washing and addition of diluted secondary antibody.
4. Develop the blot using the chromogenic substrate and view the bands.

Preparing Solutions

For western blots from NuPAGE® gels, prepare the solutions as described in the table below.

Solution	For PVDF Membrane	
Blocking Solution	Ultra filtered Water	5 ml
	Blocker/Diluent (Part A)	2 ml
	Blocker/Diluent (Part B)	3 ml
	Total Volume	10 ml
Primary Antibody Diluent	Ultra filtered Water	7 ml
	Blocker/Diluent (Part A)	2 ml
	Blocker/Diluent (Part B)	1 ml
	Total Volume	10 ml
	Dilute your primary antibody into this diluent according to the manufacturer's recommendations. Typically, primary antibody preparations are diluted 1:1000 to 1:5000 to a concentration of about 1-0.2 µg/ml.	
Antibody Wash	Ultra filtered Water	150 ml
	Antibody Wash Solution (16X)	10 ml
	Total Volume	160 ml

Continued on next page

WesternBreeze® Chromogenic Immunodetection Protocol, Continued

Protocol

1. After electrophoresis, wash the membrane twice with deionized water for 5 minutes each time.
2. Place the membrane in 10 ml Blocking Solution in a covered, plastic dish provided in the kit. Incubate for 30 minutes on a rotary shaker set at 1 revolution/second. Decant the Blocking Solution.
3. Rinse the membrane with 20 ml deionized water for 5 minutes, then decant. Repeat once.
4. Incubate the membrane with 10 ml Primary Antibody Solution for 1 hour, then decant.
5. Wash the membrane for 5 minutes with 20 ml 1X Antibody Wash, then decant. Repeat 3 times.
6. Incubate the membrane in 10 ml Secondary Antibody Solution for 30 minutes, then decant.
7. Wash the membrane for 5 minutes with 20 ml 1X Antibody Wash, then decant. Repeat 3 times.
8. Rinse the membrane with 20 ml deionized water for 2 minutes, then decant. Repeat twice.
9. Incubate the membrane in 5 ml Chromogenic Substrate until purple bands develop on the membrane. Development is complete in 1-60 minutes.
10. Rinse the membrane with 20 ml deionized water for 2 minutes. Repeat twice.
11. Air-dry the membrane on a clean piece of filter paper or dry the membrane by a stream of slightly warm air, or under an infrared lamp.

To perform stripping and reprobing or reprobing only, see page 31.

See next page for an example of expected results.

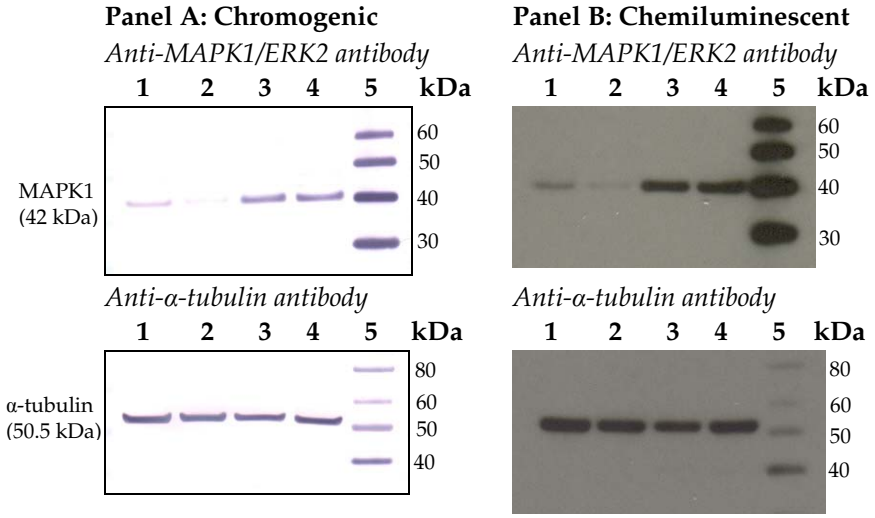
Expected Results

Example of Results

An example of results obtained after performing SDS-PAGE, western blotting, and western immunodetection are shown below. This experiment demonstrates the use of Western Analysis to validate an RNAi experiment.

In this experiment, A549 cells were transfected with Validated Stealth™ RNAi (2 different duplexes) targeting MAP Kinase 1 (MAPK1) or with Stealth™ RNAi Negative Control (medium GC) using Lipofectamine™ 2000 Reagent for transfection. 48 hours post-transfection, cells were harvested and lysed with Cell Extraction Buffer containing the Protease Inhibitor Cocktail. The lysate (8 µg) was analyzed on a NuPAGE® Novex Bis-Tris Gel and proteins were transferred onto an Invitrolon™ PVDF membrane as described in this manual. The blots were analyzed using the WesternBreeze® Chromogenic Kit (A) or Chemiluminescent Kit (B) using a polyclonal rabbit anti-MAPK1/ERK2 antibody (cat. no. 71-1800) at 1:1500 dilution or a monoclonal mouse anti-α-tubulin antibody (cat. no. 32-2500) at a 1:2000 dilution.

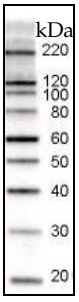
The results indicate knockdown of MAPK1 by Validated Stealth™ RNAi and show easy visualization of MagicMark™ XP Western Protein Standard on the same blot without using any special reagents.



Lane 1: Validated Stealth™ RNAi duplex 1
Lane 2: Validated Stealth™ RNAi duplex 2
Lane 3: Stealth™ RNAi Negative Control (medium GC)
Lane 4: Control (non-transfected A549 cells)
Lane 5: 5 µl MagicMark™ XP Western Protein Standard (note the difference in band intensity for the standard is due to differences in exposure times)

MagicMark™ XP Results

5 µl of MagicMark™ XP Western Protein Standard was loaded on a NuPAGE® Novex 4-12% Bis-Tris Gel, blotted onto a membrane, and detected using the WesternBreeze® Chemiluminescent Kit. The expected molecular weight for each standard band is listed.



Troubleshooting

Electrophoresis

Review the information below to troubleshoot your experiments using the XCell *SureLock*™ Mini-Cell.

Problem	Cause	Solution
Run taking longer than usual	Buffers are too dilute	Check buffer recipe; re-make if necessary.
	Upper Buffer Chamber is leaking	• Make sure the buffer core is firmly seated. • If you are using the Buffer Dam, make sure the dam is properly positioned in the core. If the core gasket is damaged, replace with a fresh gasket. • Check to ensure that the Gel Tension Wedge is in the locked position
	Voltage is set too low.	Set correct voltage as listed on page 17.
Current reading on power supply is zero or very low	Tape left on the bottom of the cassette	Remove tape from bottom of cassette.
	Connection to power supply not complete	Check all connections with a voltmeter for conductance.
	Insufficient buffer level	Make sure the buffer in the Upper Buffer Chamber (cathode) is covering the wells. Be sure there is sufficient buffer (up to the fill line) in the Lower Buffer Chamber.
	Buffer is leaking from the Upper Buffer Chamber	If the level of running buffer drops, the electrophoresis core and cassettes are not properly seated. Repeat the assembly. Check to ensure the Gel Tension Wedge is in the locked position.
Run is faster than normal with poor resolution	Buffers are too concentrated or incorrect.	Check buffer recipe; dilute or re-make if necessary.
	Voltage, current, or wattage is set at a higher limit	Decrease power conditions to the recommended run conditions (page 17).

Continued on next page

Troubleshooting, Continued

Western Blotting

Review the information provided below to troubleshoot your Western blotting experiments.

Problem	Cause	Solution
No proteins transferred to the membrane	Gel/membrane sandwich assembled in a reverse direction (proteins have migrated out)	Assemble the sandwich in the correct order using instructions provided on page 21.
Significant amount of protein is passing through the membrane indicated by the presence of proteins on the second membrane	Longer transfer time, inappropriate gel type, SDS or methanol content, or sample overloaded	• Re-evaluate the percentage of the gel used. • Shorten the transfer time by 15 minute increments. • Remove any SDS which may have been added to the transfer buffer. • Add additional methanol to increase the binding capacity of the membrane. • Decrease the sample load.
Significant amount of protein remains in the gel indicated by staining of the gel after transfer	Shorter transfer time, inappropriate gel type, SDS or methanol content Higher molecular weight proteins usually do not transfer completely as compared to mid to low molecular weight proteins	• Switch to a more appropriate lower percentage gel. • Increase the blotting time by 15 minute increments. • Add 0.01-0.02% SDS to the transfer buffer to allow migration of the protein out of the gel. • Decrease the amount of methanol in the transfer buffer.
The pH of the transfer buffer deviates from the required value by 0.2 pH units	Buffer not made up properly	Remake the buffer after checking the reagents and water quality. Do not adjust the pH with acid or base as this will increase the conductivity of the buffer and result in higher current during transfer.
Current is much higher than the expected current	Concentrated buffer used	Dilute the buffer as described on page 20.
Current is much lower than the expected start current	Very dilute buffer used resulting in increased resistance and low current	Remake the transfer buffer correctly.
	The circuit is broken (broken electrode)	Check the blot module to ensure that the electrodes are intact.
	Leak in the blot module indicated by a decrease in the buffer volume in the module	Be sure to assemble the blot module correctly to prevent any leaking.

Continued on next page

Troubleshooting, Continued

Western Blotting, continued

Problem	Cause	Solution
Power supply shuts off using recommended blotting conditions	High ionic strength of the transfer buffer	Prepare the buffer as described on page 20.
	Power supply is operating at a current close to the current limit of the power supply	Use a power supply with higher limits.
Diffuse bands and swirling pattern on the membrane	Poor contact between the gel and the membrane	Roll over the surface of each layer of the gel/membrane sandwich with a roller to ensure good contact between the gel and the membrane. Saturate the blotting pads with transfer buffer to remove air bubbles.
	Under or overcompression of the gel	Add or remove blotting pads to prevent any type of compression of the gel.
Empty spots on the membrane	Presence of air bubbles between the gel and the membrane preventing the transfer of proteins	Be sure to remove all air bubbles between the gel and membrane using a roller.
	Expired or creased membranes used	Use fresh, undamaged membranes.
Poor transfer efficiency	PVDF membrane not treated properly before use	Be sure that the membrane is pre-wetted with methanol or ethanol.
	Poor contact between the membrane and the gel	Use more blotting pads or replace the old blotting pads with new ones.
	Overcompression of the gel indicated by a flattened gel	Remove enough blotting pads so that the unit can be closed without exerting pressure on the gel and the membrane.
High background on western blots	Insufficient blocking of non-specific sites	Increase the incubation time of the blocking step.

Continued on next page

Immunodetection

Review the information below to troubleshoot your experiments using the WesternBreeze Immunodetection Kits.

Problem	Cause	Solution
High background	Membrane not completely wetted	Follow instructions for pre-wetting the membrane. Use an incubation dish which is small enough to allow thorough coverage of membrane to prevent drying out. Shake or agitate during each step.
	Membrane is contaminated	Use only clean, new membranes. Wear clean gloves at all times and use forceps when handling membranes.
	Blocking time or washing time is too short	Make sure that each step is performed for the specified amount of time. Strictly adhere to all wash times.
	Solutions or incubation tray is contaminated	Use clean glassware and purified water to prepare solutions. Replace or clean the tray thoroughly with a glassware-cleaning detergent. Rinse thoroughly with purified water. Wear clean gloves at all times.
	Concentrated Primary antibody used	Follow the supplier's recommended dilution or determine the optimum concentration by dot-blotting.
	Blot is overdeveloped, film overexposed, or became wet during exposure	Follow recommended developing time or remove blot from substrate when signal-to-noise ratio is acceptable. Decrease exposure time or allow signal to further decay. Prevent leakage by encasing membrane in transparency film and blotting excess substrate from edges before exposure.
	Multiple low molecular weight bands observed	Protein is degraded. Use protease inhibitor cocktail (added **fresh** to the Cell Extraction Buffer) during sample preparation. Increase the amount of the protease inhibitor cocktail (see page 10).
Non-Specific Binding	Membrane contaminated by fingerprints or keratin proteins	Wear clean gloves at all times and use forceps when handling membranes. Always handle membranes around the edges.
	Primary antibody too concentrated	Follow the supplier's recommended dilution or determine the optimum concentration by dot-blotting.
	Insufficient removal of SDS/weakly bound proteins from membrane after blotting	Follow instructions for membrane preparation before immunodetection.
	Short blocking time or long washing time	Make sure that each step is performed for the specified amount of time.
	Affinity of the primary antibody for the protein standards	Check with the protein standard manufacturer for homologies with primary antibody.

Continued on next page

Immunodetection, continued

Problem	Cause	Solution
Weak or no signal	Poor or incomplete transfer	Refer to troubleshooting Western Blotting (page 37) and repeat blot. After blotting, stain membrane to measure transfer efficiency. Use positive control or molecular weight marker.
	Membrane not completely wet	Follow instructions for pre-wetting the membrane. Use the Incubation Tray to allow thorough coverage of membrane to prevent drying out. Shake or agitate during each step.
	Primary antibody concentration too low, inactive primary antibody used, or low affinity of primary antibody to antigen. Antibody recognizes native protein only.	Follow the supplier's recommended dilution or determine the optimum concentration and activity by dot-blotting. Use a higher affinity primary antibody. Determine activity by performing a dot-blot. Ensure the antibody recognizes denatured protein epitope.
	Contaminated secondary antibody solution	Wear gloves at all times and keep bottles tightly capped when not in use. Use only purified water when preparing reagents.
	Protein of interest ran off the gel or poor retention of proteins	Match gel separation range to size of protein being transferred. Larger proteins require more transfer time, smaller proteins require less time.
	Sample improperly prepared; antigenicity weakened, or destroyed	SDS and reducing agents may interfere with some antibody/antigen affinities.
	Sample too dilute	Load a higher concentration or amount of protein onto the gel.
	Protein weakly bound to membrane	Ensure that transfer buffer contains 10-20% methanol.
	Insufficient substrate incubation	Perform each step for the specified amount of time or remove blot from substrate when signal-to-noise ratio is acceptable.
	Substrate is contaminated	Wear gloves at all times and keep bottles tightly capped when not in use
	Blots are too old Protein degraded	Use freshly prepared blots. Use protease inhibitor cocktail (added **fresh** to the Cell Extraction Buffer) during sample preparation. Increase the amount of the protease inhibitor cocktail (page 10).
	Insufficient exposure time (chemiluminescent kit)	Re-expose film for a longer period of time.

Appendix

Technical Service

Web Resources

Visit the Invitrogen Web site at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our web page (www.invitrogen.com).

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To request an MSDS, visit our Web site at www.invitrogen.com. On the home page, go to 'Technical Resources', select 'MSDS', and follow instructions on the page.

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Product Qualification

Introduction	Invitrogen qualifies the BioModule™ Western Analysis Unit components as described below.
MagicMark™ XP Western Protein Standard	The MagicMark™ XP Western Protein Standard is qualified on a 10% NuPAGE® Novex Bis-Tris Gel using the MOPS buffer system. After staining with Coomassie® Brilliant Blue R-250, sharp protein bands of the appropriate molecular weight must be observed. After Western blotting on nitrocellulose membranes, all protein bands must be detected using WesternBreeze® Chromogenic or Chemiluminescent Kit, Anti-Rabbit.
SeeBlue® Plus2 Pre-stained Protein Standard	The SeeBlue® Plus2 Pre-stained Protein Standard is qualified on a Novex® 10-20% Tris-Glycine or NuPAGE® Novex 10% Bis-Tris Gels using the appropriate buffer system. After electrophoresis, ten sharp protein bands of the appropriate molecular weight must be observed.
NuPAGE® Novex Bis-Tris Mini Gels	The NuPAGE® Novex Bis-Tris Mini Gels are qualified by electrophoresis of protein standards on the gels using the standard run conditions described in this manual. After electrophoresis the gels are examined for proper migration and straightness of the protein bands. The gels must be free of bubbles and debris, and the band migration must be sharp and flat.
Pre-made Buffers	The NuPAGE® LDS Sample Buffer, NuPAGE® Sample Reducing Agent, NuPAGE® MES SDS Running Buffer, and NuPAGE® Transfer Buffer are performance tested and qualified by pH and conductivity measurements. The buffers must meet the set specifications.
Invitrolon™ PVDF	Seven to nine membranes from each lot are stained for 5 minutes in 0.1%Coomassie® Blue in deionized water. After staining, the membranes are rinsed in deionized water for 15 minutes and air-dried. Membranes are inspected visually for non-specific blue marks. All sample membranes must pass inspection.

Continued on next page

WesternBreeze® Immunodetection Kit

The WesternBreeze® Chromogenic and Chemiluminescent Immunodetection Kits are qualified as follows:

10 -100 pg human IgG in NuPAGE® LDS sample buffer is electrophoresed on a NuPAGE® Novex 4-12% Bis-Tris Gel using NuPAGE® MES SDS Running Buffer. After electrophoresis, proteins are transferred onto a nitrocellulose membrane using NuPAGE® Transfer Buffer. Immunodetection is performed as described in this manual using anti-human IgG primary antibody. The amount of antigen as shown in the table below must be detected within 30-40 minutes of development.

Reagent	Amount
Rabbit	10 pg
Mouse	100 pg

Accessory Products

Additional Products

Additional equipment and reagents that may be used for electrophoresis and blotting of proteins are available separately from Invitrogen. Ordering information is provided below. For more information, visit our web site at www.invitrogen.com or call Technical Service (page 41).

A large variety of NuPAGE® Novex Mini Gels are available, see www.invitrogen.com for details.

Electrophoresis and Blotting Products	Quantity	Catalog no.
XCell *SureLock*™ Mini-Cell	1	EI0001
XCell II™ Blot Module	1	EI9051
XCell *SureLock*™ Mini-Cell with XCell II™ Blot Module	1	EI0002
Incubation Tray	8 trays	LC2102
Blotting Roller	1	LC2100
ZOOM® Dual Power Supply (100-120 VAC, 50/60 Hz)	1	ZP10001
ZOOM® Dual Power Supply (220-240 VAC, 50/60 Hz)	1	ZP10002
PowerEase® 500 Power Supply (100-120 VAC, 50/60 Hz)	1	EI8600
PowerEase® 500 Power Supply (220/240 VAC, 50/60 Hz)	1	EI8700
Pre-Mixed Buffers		
NuPAGE® LDS Sample Buffer (4X)	10 ml	NP0007
NuPAGE® Sample Reducing Agent (10X)	250 µl	NP0004
	10 ml	NP0009
NuPAGE® Antioxidant	15 ml	NP0005
NuPAGE® MES SDS Running Buffer (20X)	500 ml	NP0002
NuPAGE® Transfer Buffer (20X)	125 ml	NP0006
Reagents		
Anti-alpha Tubulin Antibody	200 µl	32-2500
Quant-iT™ Protein Assay Kit	1 kit	Q-33210
SeeBlue® Plus2 Pre-stained Protein Standard	500 µl	LC5925
MagicMark™ XP Western Protein Standard	250 µl	LC5602
Phosphate Buffered Saline (PBS), 1X	500 ml	10010-023
SimplyBlue™ SafeStain	1 L	LC6060
SYPRO® Ruby Blot Stain	200 ml	S-11791

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Continued on next page

Purchaser Notification, Continued

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